Transgenic Expression of CXCR3 Worsens the Inadequate Response to *Leishmania major* Parasites by BALB/c Mice

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Abstract

CXCR3 is a chemokine receptor present on mature Th1 cells and has been shown to be critical in the immune response to *Leishmania major* by C57BL/6 mice. Recent studies have demonstrated that when stimulated, T cells from susceptible BALB/c mice do not increase expression of CXCR3 as quickly or efficiently as resistant C57BL/6 mice. In light of these findings, this study sought to determine how transgenic overexpression of CXCR3 on T cells affects susceptibility to *L. major* infection in BALB/c mice. Contrary to our hypothesis, CXCR3-transgenic mice were more susceptible to *L.* major and did not resolve their infections. These mice instead developed a more pronounced Th2 response and developed larger lesions compared to their wild-type littermates. Concentrations of the Th1-associated IFN-γ were lowered in the draining lymph nodes of CXCR3-transgenic mice, while concentrations of the Th2-associated IL-4 and the immunomodulatory IL-10 were significantly elevated, thus contributing to parasite persistence. Other immune cell types including neutrophils and monocytes were heavily affected by the immunological imbalances caused by expression of the CXCR3 transgene. The accumulation of these cells may serve to explain why $CXCR3^{Tg}$ mice were unable to control the parasite. This study shows that overexpression of CXCR3 is insufficient to confer resistance upon BALB/c mice following infection with *L. major*.

Introduction

Organisms of the genus *Leishmania* are protozoan parasites which are found on every continent except Antarctica. These parasites are spread by bites of the female sand fly and are the causative agent of leishmaniasis. Leishmaniasis manifests clinically in different forms depending on the infective species of parasite. One of the most common forms of leishmaniasis is cutaneous leishmaniasis (CL), which is caused by several species of *Leishmania*, including *Leishmania major.* CL is characterized by the formation of a progressive lesion which destroys tissue at the site of infection. After the initial growth of

the parasite, CL frequently subsides in human hosts and the lesion scars over. Other forms of the disease are characterized by the movement of parasites beyond the initial infection site to mucosal membranes or visceral organs such as the liver or spleen. These forms are more severe and lead to significant disfigurement and mortality if left untreated. Leishmaniasis is currently one of the largest neglected tropical diseases, with 1.5-2 million new infections per year and approximately 59,000 deaths, which can be mostly attributed to the visceral form of the disease [1].

Leishmania parasites are remarkable because of their ability to survive inside of phagocytic cells including neutrophils and macrophages. Upon introduction of *Leishmania* parasites through a sand fly bite wound, the parasites are mainly taken up through phagocytosis by neutrophils [2]. Despite the fact that neutrophils are some of the most potent killers of foreign pathogens in the human body, the parasite persists in these cells and rapidly switches from its infective promastigote form to its replicative amastigote form. For most pathogens, phagocytosis is a death sentence. Phagocytes such as macrophages and neutrophils are able to produce reactive oxygen and nitrogen species (ROS and RNS, respectively) and acidify the parasitophorous vacuole [3]. These molceules are highly toxic to living cells and therefore represent a considerable obstacle for potential pathogens. For *Leishmania*, however, the parasite is able to withstand and interfere with the antimicrobial activities of these immune cells. Following apoptosis by infected neutrophils, amastigotes are then taken up by macrophages, which are the preferred host for the parasite. The amastigotes are then able to replicate inside of the macrophage until it bursts, at which point the parasite can spread to other macrophages and persist for long periods of time in the areas surrounding the initial bite wound [2]. The life cycle is completed when the parasite is picked up by another sand fly during a blood meal and is able to begin replicating and preparing to infect its next host.

The parasite's ability to evade efficient killing by innate immune cells makes it very difficult to eradicate from the host. The adaptive response to *Leishmania* is therefore paramount in controlling the parasite. While the normal vacuolar activity of macrophages is often insufficient to kill the parasite, inflammatory activation by T cells drastically increases the leishmanicidal activity of these cells [4]. In mouse models of CL, a clear dichotomy exists between two different subsets of helper T cells, Th1 and Th2. A polarized Th1 immune response favors resistance to the parasite and a polarized Th2 response favors susceptibility [5]. The Th1 response is characterized by the production and accumulation of cytokines such as IFN-γ, IL-12, and TNF-α, which promote inflammation at the lesion as well as classical activation of macrophages, which increases the production of reactive nitrogen species (RNS) and facilitates the killing of the parasite [6]. The Th2 response, which is associated with the cytokines IL-4, and IL-13, favors alternative activation of macrophages and increases the activity of eosinophils and B cells. The actions of these cell types are primarily geared toward the elimination of extracellular parasites, with eosinophils capable of releasing lytic granules which can facilitate parasite degradation, and B cells producing large amounts of parasite-specific antibodies [7]. The actions of these cells are insufficient to control the growth of an intracellular parasite such as *Leishmania*, mainly due to the fact that many of the effector molecules generated by this response are limited to the extracellular space. In addition to being unable to effectively target the parasite, the Th2 response also favors alternative, rather than classical, activation of the macrophage, meaning that macrophages do not assume an inflammatory role and perform a more regenerative role which is not conducive to parasite killing.

Importantly, the Th1 and Th2 responses oppose one another. Th1 cytokines inhibit the production or activity of Th2 cytokines, and vice versa. In the context of leishmaniasis, this makes a Th2 response harmful for two reasons. Firstly, the Th2 response is largely ineffective at directly clearing the parasite, which remains obscured from extracellular effector molecules due to its intracellular life cycle inside of the host. Secondly, the Th2 response opposes the IFN-γ-mediated Th1 response which is essential for

clearance of the parasite. Because of this clear Th1-Th2 dichotomy, leishmaniasis has been used extensively as a means for studying the unique roles of the Th1 and Th2 helper T cell subtypes.

In murine models of *L. major*, this dichotomy can be clearly demonstrated in different genetic backgrounds. C57BL/6 mice, which develop a strong Th1 response, are resistant to the parasite and resolve lesions caused by *L. major*. BALB/c mice, however, mount a Th2 response and develop progressive, uncontrolled lesions. The reasons for this difference in resistance have yet to be elucidated, but some studies have speculated that inefficient expression of the IL-12 receptor on the surfaces of T cells in BALB/c mice could be a main reason for this susceptibility [8] [9] [10]. This conclusion, however, is currently disputed, as transgenic expression of the IL-12 receptor on T cells was insufficient to confer resistance upon BALB/c mice [11]. It is likely that a lack of IL-12 responsiveness is one of many factors which contributes to BALB/c susceptibility, so recent studies have sought to investigate other factors, such as the role of chemokines and their receptors in the context of murine CL. Chemokines and chemokine receptors are responsible for the efficient trafficking, through chemotaxis, of immune cells to different locations around the body, and as such, their role is critical in the context of leishmaniasis. Of special importance are chemokine receptors on T cells, which allow the T cell to follow molecular gradients toward infection sites and allow it to exert its function at the correct location. One such chemokine receptor is CXCR3, which responds to the ligands CXCL9, CXCL10, and CXCL11 and is present on activated Th1 cells [12]. CXCR3 therefore has a critical role in guiding Th1 cells to the infection site. The trafficking of these cells is one of the most important steps in the immune response to *L. major,* as Th1 cells are chiefly responsible for the production of IFN-γ, and this molecule is necessary to activate macrophages and facilitate killing of the parasite.

Recent studies have underscored the importance of this CXCR3-dependent Th1 trafficking *in vivo*. CXCR3 has been demonstrated to be crucial in maintaining resistance to *L. major*, as C57BL/6 mice lose their

protective immunity when CXCR3 is knocked out [13]. Importantly, these mice maintain their Th1 phenotype but are unable to control the parasite due to the interruption of normal Th1 cell trafficking. Investigations of CXCR3 in *L. major*-susceptible BALB/c mice also appear to emphasize this importance, as BALB/c mice have been shown to be deficient in their up-regulation of CXCR3 expression following challenge with *L. major* [14]. Together, these observations suggest that induced overexpression of CXCR3 could remedy the insufficient expression of this molecule by BALB/c mice and thus increase their resistance to the parasite. This study sought to answer the question of whether CXCR3, when expressed at high levels on the T cells of BALB/c mice, could increase resistance to *L. major in vivo*.

Materials and Methods:

Generation of CXCR3 Transgenic ($CXCR3^{Tg}$) Mice:

A transgenic CXCR3 mouse was generated by Dr. Steve Oghumu via insertion of a linearized targeting vector (TV) containing CXCR3 cDNA between a human CD2 promoter and CD2 locus control region (LCR) into the pronuclei of C57BL/6 embryos. The CD2 promoter and LCR were used in order to ensure T cell specificity [15]. Screens for $CXCR3^{Tg}$ mice were conducted via a polymerase chain reaction assay (PCR) using genomic DNA isolated from ear tissue samples. This assay was conducted as previously described [16] using two separate primer sets. Primer set 1 yielded band sizes of 558bp ($CXCR3^{WT}$) and 295bp ($CXCR3^{Tg}$), while primer set 2 yielded band sizes of 267bp ($CXCR3^{WT}$) and 402bp ($CXCR3^{Tg}$). $CXCR3^{Tg}$ C57BL/6 males were identified using this assay and were bred to WT BALB/c females for a total of 10 generations to ensure that the pups used for experimental purposes would be completely genetically BALB/c in character. All $CXCR3^{Tg}$ and $CXCR3^{WT}$ mice discussed in this work should be considered genetically BALB/c unless otherwise indicated.

Experimental Mice

Experimental mice were identified as $CXCR3^{Tg}$ or $CXCR3^{WT}$ based on the PCR assay described above. 5 BALB/c WT control mice were purchased for each experiment to use as controls. All mice were maintained in a pathogen-free facility at the Ohio State University in accordance with all pertinent NIH and IACUC regulations.

Parasites and Infection Protocol:

Leishmania major amastigotes (LV39) were maintained in wild type BALB/c mice and isolated from infected lesions and grown in batch cultures of complete M199 media (Gibco, Carlsbad, California) containing 10% heat-inactivated FBS (Atlanta Biologicals, Flowery Branch, Georgia), 1% penicillin-streptomycin, and 1% HEPES (Gibco, Carlsbad, California), as previously described [17]. The amastigotes isolated from this procedure were passaged three times, inducing reversion to the maximally infectious metacyclic promastigote form. Stationary phase promastigotes were then collected by centrifugation. Infectious doses of 2.0×10^6 parasites were inoculated into the left footpads of age-matched 8 week-old mice from transgenic breeding pairs.

Footpad Measurement:

The thicknesses of both the left and right footpad were measured weekly using a micrometer (Mitutoyo, Kanagawa, Japan). Lesion sizes were determined by subtraction of the width of the uninfected right footpad from the thickness of the infected left footpad.

Quantification of parasite loads:

At week 10 post-infection, footpad lesions were isolated and grown in Schneider's media (Gibco, Carlsbad, California). Parasites were enumerated by a limiting-dilution assay as previously described [17] [18].

Cytokine ELISA and T cell proliferation assay:

Lymph nodes and spleens of infected mice were harvested under sterile conditions at week 10 post-infection. Single cell suspensions were prepared by gentle teasing in complete 1640 RPMI medium (Gibco, Carlsbad, California), which included 10% inactivated FBS, 1% penicillin-streptomycin, 1% HEPES and 500µL of β-mercaptoethanol (Gibco, Carlsbad, California). The cells were then counted and adjusted to concentrations of 5.0 X 10^6 cells/mL for the spleen and 3.0 X 10^6 cells/mL for the lymph node. These suspensions then were plated and re-stimulated with *L. major* antigen for 72 hours at 37°C. T cell proliferation was measured by an alamar blue assay as previously described [19]. The supernatants were removed from these plates and used for subsequent cytokine ELISA. Concentrations of IFN-γ, IL-4, and IL-10 in the supernatants were assayed by cytokine ELISA. This procedure was performed as previously described [19].

Antibody ELISA:

Blood samples were collected every two weeks post-infection by nicking the tail vein with a scalpel and collecting the blood in a 1.5 mL microcentrifuge tube (Fisher Scientific, Hampton, New Hampshire) to a maximum amount of 100µL. Serum was isolated by centrifugation at 13,000 RPM for 5 minutes. This serum was analyzed using IgG1- and IgG2a-specific antibodies during an enzyme-linked immunosorbent assay (ELISA) as previously described [20].

Flow cytometry:

Single-cell suspensions were isolated from lymph nodes and spleens of infected WT and $CXCR3^{Tg}$ mice. These cells were then stained for CD4, CD8, CD3, CD11b, Gr1, and F4/80 in addition to the Th1- and Th2-specific surface stains, Tim3 and ST2 (Biolegend, San Diego CA). Selected single cell suspensions were incubated for six hours in Phorbol 12-myristate 13-acetate (PMA), ionomycin, and brefeldin A (Sigma Aldrich, St. Louis MO). These suspensions were then stained using IFN-γ- and IL-4-specific intracellular

stains. Cells were acquired in a FACSCalibur flow cytometer (BD Biosciences, San Jose CA). Results from this assay were analyzed using FlowJo software (Tree Star, Inc.).

Statistical analysis:

Student's unpaired t-test was used to determine the significance of any results obtained. A t-test which yielded a p value of <0.05 was considered significant. Statistical significance of varying degrees was denoted using the following convention: *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$.

Results

Verification of CXCR3 transgene expression in CXCR3^{Tg} mice

CXCR3^{Tg} mice used in this experiment did not have any noticeable impairment in development and were viable and healthy. The transgene appeared in about 50% of pups born to transgenic breeders. PCR screens were used to identify CXCR3^{WT} and CXCR3^{Tg} mice from each litter (Figure 1). Primer set 1 yielded band sizes of 295 bp (CXCR3^{Tg}) and 558bp (CXCR3^{WT}), while primer set 2 yielded band sizes of 267bp (CXCR3^{WT}) and 402bp (CXCR3^{Tg}). In order to verify overexpression of CXCR3 on the surface of T cells from CXCR3^{Tg} mice, a flow cytometric analysis was conducted on single-cell suspensions of thymuses, lymph-nodes, and spleens of pups from C57BL/6 and BALB/c CXCR3^{Tg} breeders. T cell-specific expression of CXCR3 was universal among CXCR3^{Tg} mice, whereas their CXCR3^{WT} littermates expressed CXCR3 at much lower rates (Figure 2). This trend was verified during active CL with universal expression of CXCR3 on CD4^{+} T cells following infection with *L. major* (Figure 3).

CXCR3^{Tg} mice display a worsened disease phenotype following infection with *L. major*

Contrary to our hypothesis, CXCR3^{Tg} mice displayed significantly larger footpad lesions than their CXCR3^{WT} littermates following infection with *L. major* (Figure 4). The lesion sizes of CXCR3^{Tg} mice increased more rapidly and at an earlier time point than their CXCR3^{WT} littermates. Limiting dilution

assays were undertaken to determine parasite titers, and these results confirmed the surprising trend detailed above; CXCR3^{Tg} mice had significantly larger numbers of parasites in their infected lesions (Figure 5).

Antibody class switching and T cell proliferation in response to CL were unaffected by transgenic expression of CXCR3

Despite evidence to suggest that CXCR3^{Tg} mice were unable to control *L. major* infection, no differences were detected in the titers of two representative antibody classes, IgG1 and IgG2a (Figures 6,7). These antibody classes, if present at high titers, can indicate class switching due to a Th2 or Th1 response, respectively. Surprisingly, despite the worsened disease phenotype, these results indicate that class switching during CL is largely unaffected by the overexpression of CXCR3. Additionally, alamar blue assays to quantify T cell proliferation from lymph node and spleen cell suspensions showed that T cell proliferation was also unaffected by the overexpression of CXCR3 (Figure 8). Importantly, this assay established that T cell proliferation by CXCR3^{Tg} mice was unaffected by the overexpression of CXCR3.

CXCR3^{Tg} mice mount a more pronounced Th2 response to CL than their CXCR3^{WT} littermates

While there was little evidence to suggest that antibody class switching due to a prevailing Th1 or Th2 response was altered in CXCR3^{Tg} mice, cytokine ELISA revealed that these CXCR3^{Tg} mice mounted a more pronounced Th2 response than their CXCR3^{WT} littermates, a trend which was not reflected in antibody titers. Concentrations of the canonical Th1 cytokine, IFN-γ, were significantly reduced in the draining lymph nodes of CXCR3^{Tg} mice, while concentrations of the Th2-associated IL-4 and the immunomodulatory molecule IL-10 were found to be significantly increased in the lymph nodes of these mice (Figure 9). The Th2 bias which develops naturally in response to *L. major* in BALB/c mice appeared to be enhanced in mice which overexpressed CXCR3. To verify this trend, intracellular stains for IFN-γ and IL-4 were used to determine percentages of IFN-γ- and IL-4-producing T cells in draining lymph

nodes. While the results were not significant, percentages of IFN-γ- and IL-4-producing T cells reflected the same trends observed by cytokine ELISA. $CXCR3^{Tg}$ mice had lower percentages of IFN-$\gamma^{+}CD4^{+}$ T cells and higher percentages of IL-4^{+} $CD4^{+}$ T cells than their $CXCR3^{WT}$ littermates (Figure 10). A direct measurement of the Th1:Th2 balance was performed using flow cytometry with the Th1- and Th2-specific stains, Tim-3 and ST2. These direct measurements of Th1 and Th2 cell accumulation showed the same trends as discussed above, with $CXCR3^{Tg}$ mice having lower percentages of Th1 cells and higher percentages of Th2 cells in their draining lymph nodes (Figure 11).

$CXCR3^{Tg}$ expression affects monocyte and neutrophil accumulation and maturation

While neutrophils and monocytes do not express CXCR3 and would not be expected to accumulate differently in $CXCR3^{Tg}$ mice, flow cytometry revealed that *L. major*-infected $CXCR3^{Tg}$ mice have significantly altered populations of both cell types in their draining lymph nodes. Populations of these cells were quite apparent in $CXCR3^{Tg}$ mice upon gating (Figure 12), and calculations to determine the total numbers revealed that the accumulation of these cells was indeed extremely high in $CXCR3^{Tg}$ mice (Figure 13). Among the monocyte population, flow cytometry also revealed that many of these monocytes had not yet matured. Once again, this was quite apparent by looking at gated populations of monocytes using F4/80 as a marker of maturity (Figure 14). Overall percentages revealed a significant difference in monocyte maturity between $CXCR3^{Tg}$ mice and their $CXCR3^{WT}$ littermates (Figure 15).

Discussion

Given CXCR3's importance to the adaptive immune response, it is unsurprising that interventions which affect its expression can have significant consequences in the context of leishmaniasis. What is surprising, however, is that overexpression of CXCR3 in this experiment not only failed to decrease susceptibility among BALB/c mice, but indeed that it severely worsened it. This increased susceptibility was observed alongside a large increase in Th2 cytokine production and cell accumulation in transgenic

mice. These findings were completely unexpected because of what has been shown about CXCR3 deficiencies in the context of CL. Recent work by our lab concluded that C57BL/6 CXCR3 -/- mice lose their protective immunity to *L. major* [13]. Importantly, this study also concluded that CXCR3 -/- mice retained a distinctive Th1 phenotype regardless of CXCR3 expression. Another study of a transcription factor which lies upstream of CXCR3 in the Th1 cell development pathway, STAT1, concluded that overall Th1 differentiation in response to *L. major* was unaffected, but noted that the observed susceptibility to the parasite was due to an inability to up-regulate CXCR3 expression [19]. One of the unifying findings of these experiments was that CXCR3 played little to no role in Th1-Th2 differentiation; instead, these studies indicated that the only major deficiencies in STAT1 or CXCR3 -/- mice were in the area of T cell migration to the site of infection.

What we show, here, however, is that CXCR3 overexpression has significantly affected the Th1-Th2 balance, something that did not appear to happen in knockout studies. Another unifying aspect of these studies was that they did not interfere with factors which could significantly alter the Th1-Th2 balance. In murine CL, the early signals which control Th1 or Th2 response development are exceedingly important. In BALB/c mice infected with *L. major*, the early production and response to the Th1-associated cytokine IL-12 has been examined closely. IL-12 production by macrophages early in the immune response to *L. major* has been shown to be fairly consistent among various strains of mice; indeed bone marrow-derived macrophage populations in BALB/c mice produced *more* of the IL-12 p40 subunit than C57BL/6 mice in one study [21]. Despite this production, BALB/c mice still develop a strong Th2 immune response. The early induction of IL-4 and subsequent down-regulation of IL-12R expression have often been blamed for the development of the ineffective Th2 response to *L. major* by WT BALB/c mice, and some studies have given this theory credence [9][22][23][24]. This model of lowered IL-12 sensitivity by down-regulation of the IL-12R provides a potential mechanism for the observed increase in susceptibility by $CXCR3^{Tg}$ mice, as the cellular machinery which allows for the quick induction of a Th2

response was likely unaffected by the overexpression of CXCR3. Since the CXCR3 transgene used in this experiment was T cell-specific but not Th1-specific, this meant that other subsets of T cells, including regulatory T cells, naïve T cells, and Th2 cells, also expressed CXCR3 and likely migrated to the infection site in greater numbers than normal. The naïve T cells of $CXCR3^{Tg}$ mice expressed CXCR3, but also retained their predisposition toward Th2 differentiation as discussed above, and as such, larger amounts of Th2 cytokines and Th2 cells were observed. Importantly, Th2 cells oppose the development of a Th1 response and were likely responsible for further dampening the already-inadequate production of IFN-γ by BALB/c mice (Figures 9,10). Additionally, IL-10 production, which suppresses inflammation and is associated with parasite persistence, appeared to be enhanced in $CXCR3^{Tg}$ mice, which could be due to abnormal accumulation of regulatory T cells as well as Th2 cells (Figure 9).

Another unexpected consequence of CXCR3 overexpression was the large increase in neutrophil and monocyte accumulation in the draining lymph nodes of infected $CXCR3^{Tg}$ mice (Figures 12-15). The role of neutrophils in the context of CL remains to be fully elucidated, but studies have shown that *Leishmania* parasites are able to withstand the harsh conditions inside of neutrophils, and thus these cells have been shown to be transient host cells for the parasite in the minutes and hours following the initial infection. The early uptake by neutrophils appears to be a crucial step for *Leishmania's* initial invasion [2]. One study goes so far as to conclude that *Leishmania* promastigotes induce the chemotaxis of neutrophils toward the infection site, further reinforcing the importance of early neutrophil uptake in the process of *Leishmania* infection [25]. *Leishmania* is unable to replicate efficiently inside of neutrophils, but some studies have indicated that the parasite uses the transient neutrophil phase of its life cycle to modulate the early immune response by skewing it toward a Th2 response and preventing efficient migration of Th1 cells to the site of infection [25][26][27].

Neutrophils have very short life spans in healthy hosts, with spontaneous apoptosis often occurring after only 6-10 hours in circulation [28]. Infection with *Leishmania* promastigotes extends this lifespan substantially by interfering with caspase-3-mediated apoptosis [29]. The role of neutrophils in the response to *Leishmania* remains controversial, but the results of these studies clearly indicate that the presence of large numbers of neutrophils in infected areas could aid the growth of the parasite. While the presence of the CXCR3 transgene likely does not directly affect the recruitment of neutrophils, the observed recruitment of these cells was likely mediated by the large numbers of parasites which resided in the lesion, as well as the significant tissue damage that the exacerbated infection caused.

The increased accumulation of monocytes was also paradoxical, as these cells do not express CXCR3 and would not be expected to be affected by CXCR3 overexpression. Monocytes are precursor cells to macrophages, meaning that their role in the immune response to *Leishmania* is extensive. The accumulation of these cells, as is the case with neutrophils, can often benefit *L. major* parasites. Monocytes, however, can mature and become activated, a process which presents a major risk to the parasite, so infection of immature monocytes and unactivated macrophages is crucial for the survival and persistence of *Leishmania* in its host [30][31][32]. The massive infiltration of monocytes into the lymph nodes of $CXCR3^{Tg}$ mice was therefore problematic, as very few of these cells were $GR1^{+}F4/80^{+}$, meaning that many of these cells were immature. Previous studies have shown that $GR1^{+}F4/80^{-}$ cells are unable to efficiently kill *L. major* parasites and are major mediators of parasite persistence [32]. The finding that many of the infiltrating monocytes in $CXCR3^{Tg}$ mice were immature could help to explain why the parasites were able to accumulate so effectively, as there was no shortage of host cells to infect with such an ample supply of immature monocytes. The large populations of immature monocytes can be explained by the significant amounts of IL-10 produced in the draining lymph nodes of $CXCR3^{Tg}$ mice. IL-10 inhibits monocyte maturation and serves primarily to modulate the immune response [33]. It is therefore unsurprising that the IL-10 production which was observed in the lymph nodes of $CXCR3^{Tg}$

mice could have led to the downstream inhibition of monocyte maturation and thus persistence of the parasite.

In conclusion, we have shown that CXCR3^{Tg} mice are unable to control infections with *L. major* and develop larger lesions with more parasites than their CXCR3^{WT} littermates. The natural susceptibility to the parasite is enhanced in CXCR3^{Tg} mice by a more pronounced Th2 response, which is accompanied by increased concentrations of Th2 cytokines and large numbers of Th2 cells. T cell proliferation and antibody class switching appeared to be unaffected by transgenic expression of CXCR3, despite the clear differences in other aspects of the immune response. Importantly, the accumulation of other immune cell populations was heavily altered, as was the case with both neutrophils and monocytes. Both of these populations were profoundly affected by expression of the CXCR3 transgene and likely contributed significantly to the increased susceptibility to *L. major* observed in CXCR3^{Tg} mice.

Figures

Figure 1: Polymerase chain reaction allows for identification of CXCR3^{Tg} mice. Genomic DNA from ear samples was prepared and analyzed via polymerase chain reaction (PCR) using two different primer sets specific for transgenic and wild-type copies of the CXCR3 gene. PCR products were visualized following gel electrophoresis in a 1% agarose gel. Bands of size 295bp (CXCR3^{Tg}) and 558bp (CXCR3^{WT}) were observed in reactions containing primer set 1, while bands of 267bp (CXCR3^{WT}) and 402bp (CXCR3^{Tg}) were observed in reactions containing primer set 2.

Figure 2: Verification of universal expression of CXCR3 on T cells of naïve mice. Single cell suspensions of thymus, lymph node, and spleen cells from CXCR3^{WT} and CXCR3^{Tg} mice in C57BL/6 (BL/6) and BALB/c (BC) backgrounds were prepared and stained using fluorescent antibodies specific for CD3, CD4, CD8, and CXCR3. CXCR3 expression by CD3^{+} cell populations was evaluated. Percentages of CXCR3^{+} cells from

gated $CD3^+$ populations are listed beside histogram plots from each tissue type. Data is representative of three experiments and similar results.

Figure 3: Verification of universal CXCR3 expression on $CD4^+$ T cells following infection with *L. major*. Flow cytometry of single cell suspensions from draining lymph nodes of *L. major*-infected BALB/c $CXCR3^{WT}$ and $CXCR3^{Tg}$ mice was performed using CD3, CD4, CD8, and CXCR3-specific stains as described above. Data is representative of three experiments with three mice per group and similar results.

Figure 4: Footpad lesion size comparison following infection with *L. major*. Footpad lesion sizes were obtained by measurement of the width of the infected left footpad minus the width of the uninfected right footpad in $CXCR3^{WT}$ and $CXCR3^{Tg}$ mice. Data is representative of mean ± SEM compiled from two separate experiments with 5 to 10 mice per group and similar results. $^{**}p < 0.01$, $^{***}p < 0.001$ using student's unpaired t test.

Figure 5: Parasite burdens of $CXCR3^{WT}$ and $CXCR3^{Tg}$ mice. Parasites were enumerated via a limiting dilution assay following isolation of the infected left footpad. Data is representative of mean ± SEM compiled from two separate experiments with 5 to 10 mice per group and similar results. $^{***}p < 0.001$ using student's unpaired t test.

Figure 6: Analysis of IgG1 in serum following infection with *L. major*. Serum was obtained and isolated by nicking of tail veins at weeks 5, 8, and 10 post-infection. This serum was analyzed by antibody ELISA to determine titers of IgG1. Data is representative of mean ± SEM for one experiment with 5 mice per group.

Figure 7: Analysis of IgG2a in serum following infection with *L. major*. Serum was obtained and isolated by nicking of tail veins at weeks 5, 8, and 10 post-infection. This serum was analyzed by antibody ELISA

to determine titers of IgG2a. Data is representative of mean ± SEM for one experiment with 5 mice per group.

Figure 8: T cell proliferation following restimulation with *L. major* antigen. T cell proliferation was evaluated following restimulation of lymph node and spleen single cell suspensions with *L. major* antigen. Data is representative of mean ± SEM for one experiment with 5 mice per group.

Figure 9: Cytokine concentrations in draining lymph nodes of *L. major*-infected mice. Cytokine concentrations were evaluated using a cytokine ELISA for IFNγ, IL-4, and IL-10. Data represents mean ± SEM compiled from two separate experiments with 5 to 10 mice per group and similar results. *$p < 0.05$, **$p < 0.01$, using student's unpaired t test.

Figure 10: Intracellular cytokine staining of $CD4^+$ T cells in draining lymph nodes. Single cell suspensions from draining lymph nodes of *L. major*-infected mice were analyzed following permeabilization by flow cytometry using stains for CD3, CD4, CD8, IFN-γ, and IL-4. Data represents mean ± SEM for one experiment with three mice per group.

Figure 11: Th1 and Th2 staining of $CD4^+$ T cells in draining lymph nodes. Single cell suspensions from draining lymph nodes of *L. major*-infected mice were analyzed by flow cytometry using stains for CD3, CD4, CD8, Tim-3 and ST2. Data represents mean ± SEM for one experiment with three mice per group. **$p < 0.01$, using student's unpaired t test.

Figure 12: Myeloid populations in draining lymph nodes. Single cell suspensions from draining lymph nodes of *L. major*-infected mice were analyzed by flow cytometry using stains for CD11b, Gr-1, CD206, and F4/80. Percentages of $GR\text{-}1^{hi}CD11b^{hi}$ and $GR\text{-}1^{int/lo}CD11b^{hi}$ gated populations are shown. Plots are representative of one experiment with three mice per group and similar results.

Figure 13: Analysis of myeloid population size in draining lymph nodes. Single cell suspensions from draining lymph nodes of *L. major*-infected mice were analyzed by flow cytometry using stains for CD11b, Gr-1, CD206, and F4/80. Data represents mean ± SEM of total population sizes for one experiment with three mice per group.

Figure 14: Evaluation of monocyte maturation by F4/80 expression. Single cell suspensions from draining lymph nodes of *L. major*-infected mice were analyzed by flow cytometry using stains for CD11b, Gr-1, CD206, and F4/80. Gated monocyte populations were analyzed for expression of F4/80. Percentages of $F4/80^{+}$ cells are shown. Plots are representative of one experiment with three mice per group and similar results.

Figure 15: Analysis of monocyte maturation in draining lymph nodes. Single cell suspensions from draining lymph nodes of *L. major*-infected mice were analyzed by flow cytometry using stains for CD11b, Gr-1, CD206, and F4/80. Data represents mean ± SEM of gated population percentages sizes for one experiment with three mice per group.

Figure 1

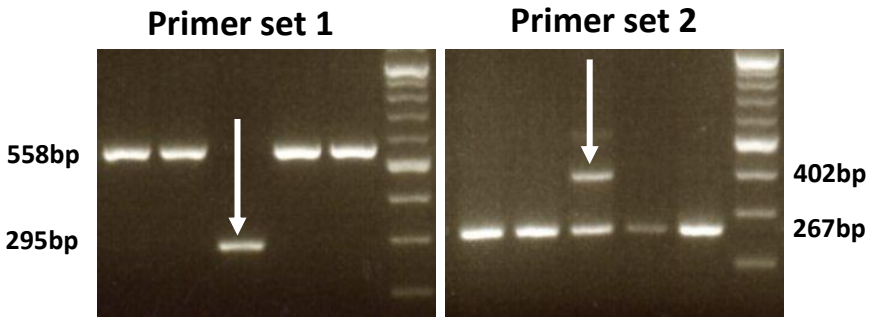


Figure 2

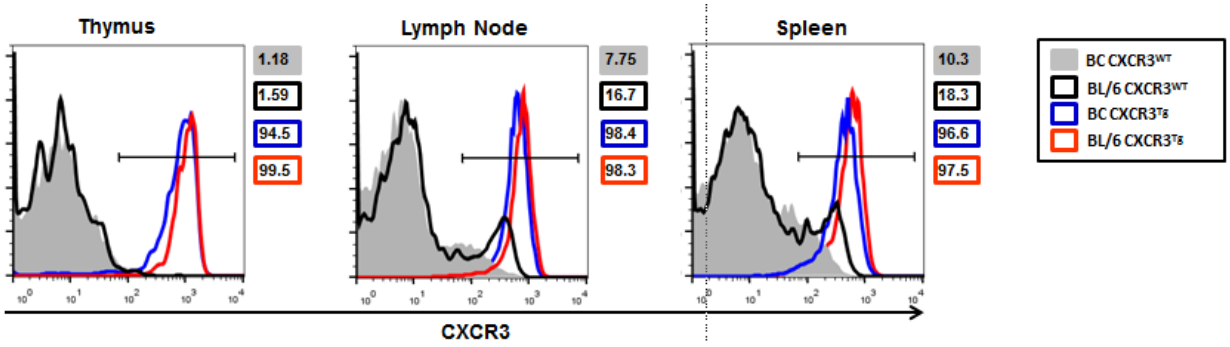


Figure 3

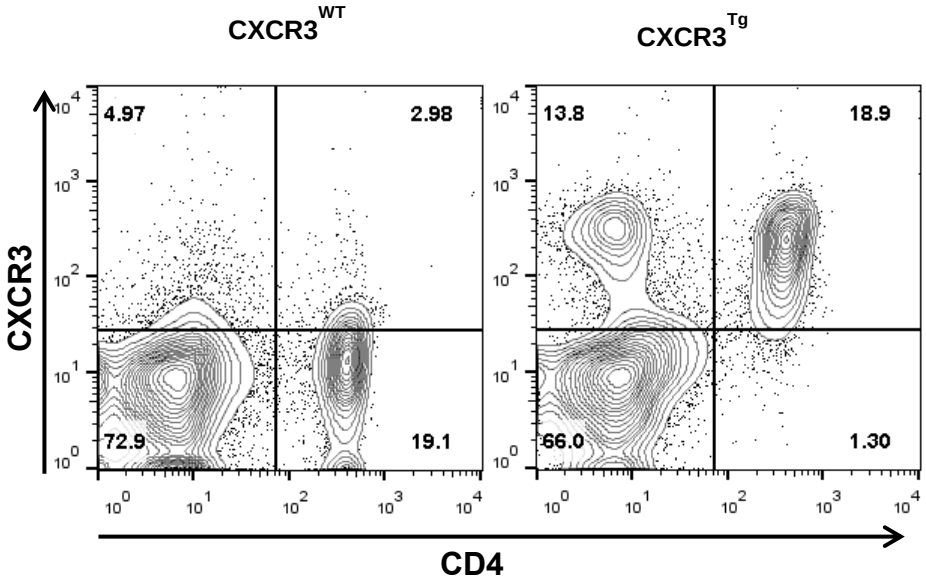


Figure 4

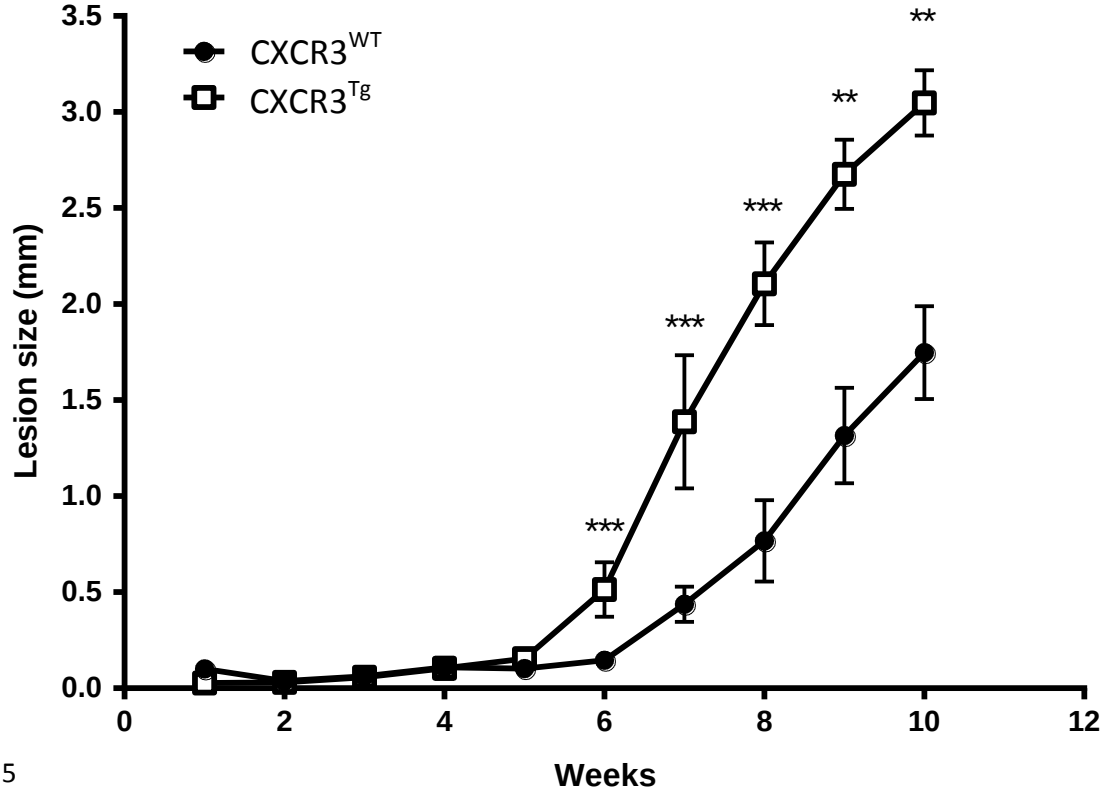


Figure 5

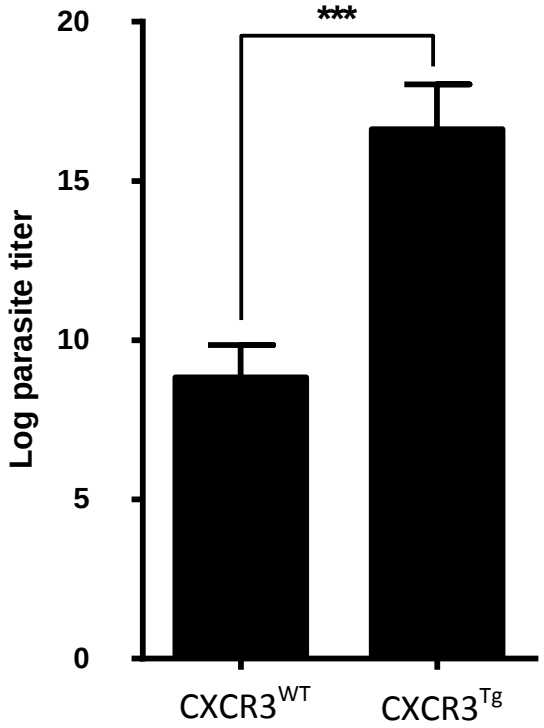


Figure 6

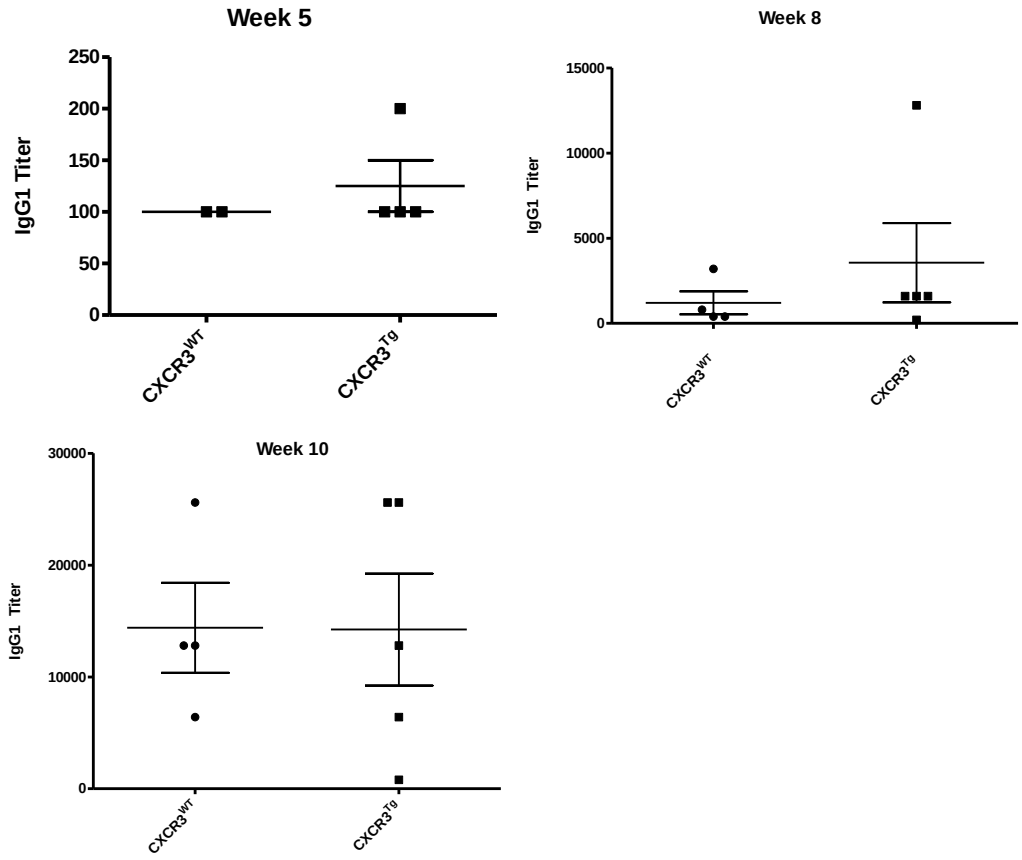

Week 5
IgG1 Titer
250
200
150
100
50
0
CXCR3WT
CXCR3Tg
Week 8
IgG1 Titer
15000
10000
5000
0
CXCR3WT
CXCR3Tg
Week 10
IgG1 Titer
30000
20000
10000
0
CXCR3WT
CXCR3Tg

Figure 7

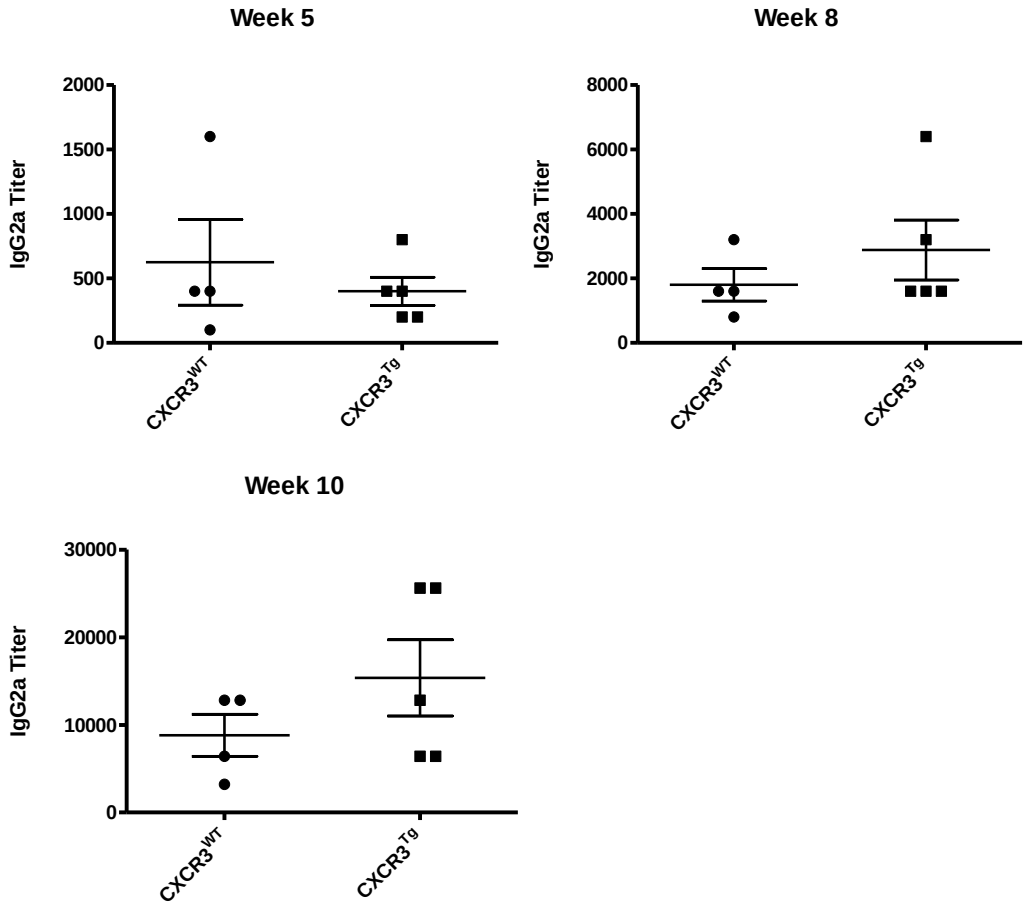
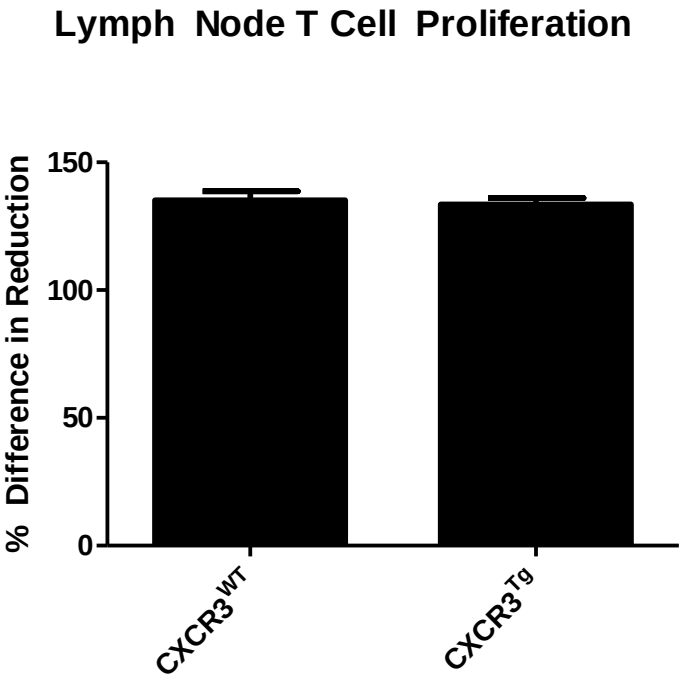

Week 5
IgG2a Titer
2000
1500
1000
500
0
CXCR3WT
CXCR3Tg
Week 8
IgG2a Titer
8000
6000
4000
2000
0
CXCR3WT
CXCR3Tg
Week 10
IgG2a Titer
30000
20000
10000
0
CXCR3WT
CXCR3Tg

Figure 8


Lymph Node T Cell Proliferation
% Difference in Reduction
150
100
50
0
CXCR3WT
CXCR3Tg

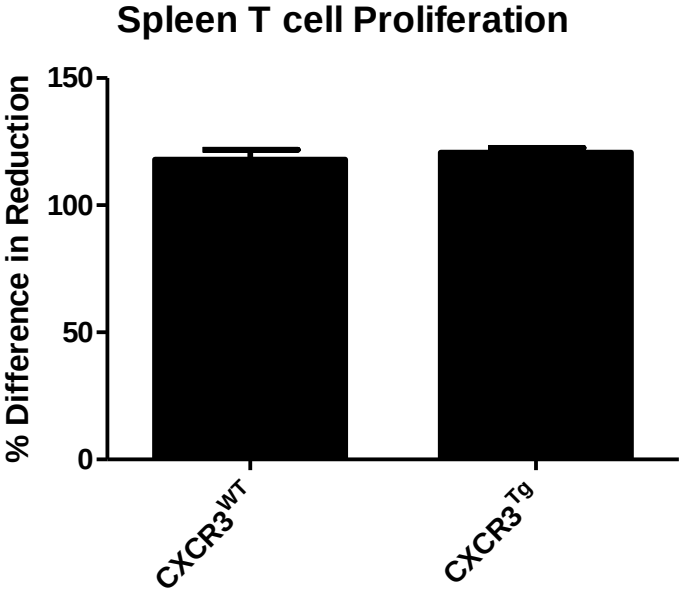

Spleen T cell Proliferation
% Difference in Reduction
150
100
50
0
CXCR3WT
CXCR3Tg

Figure 9

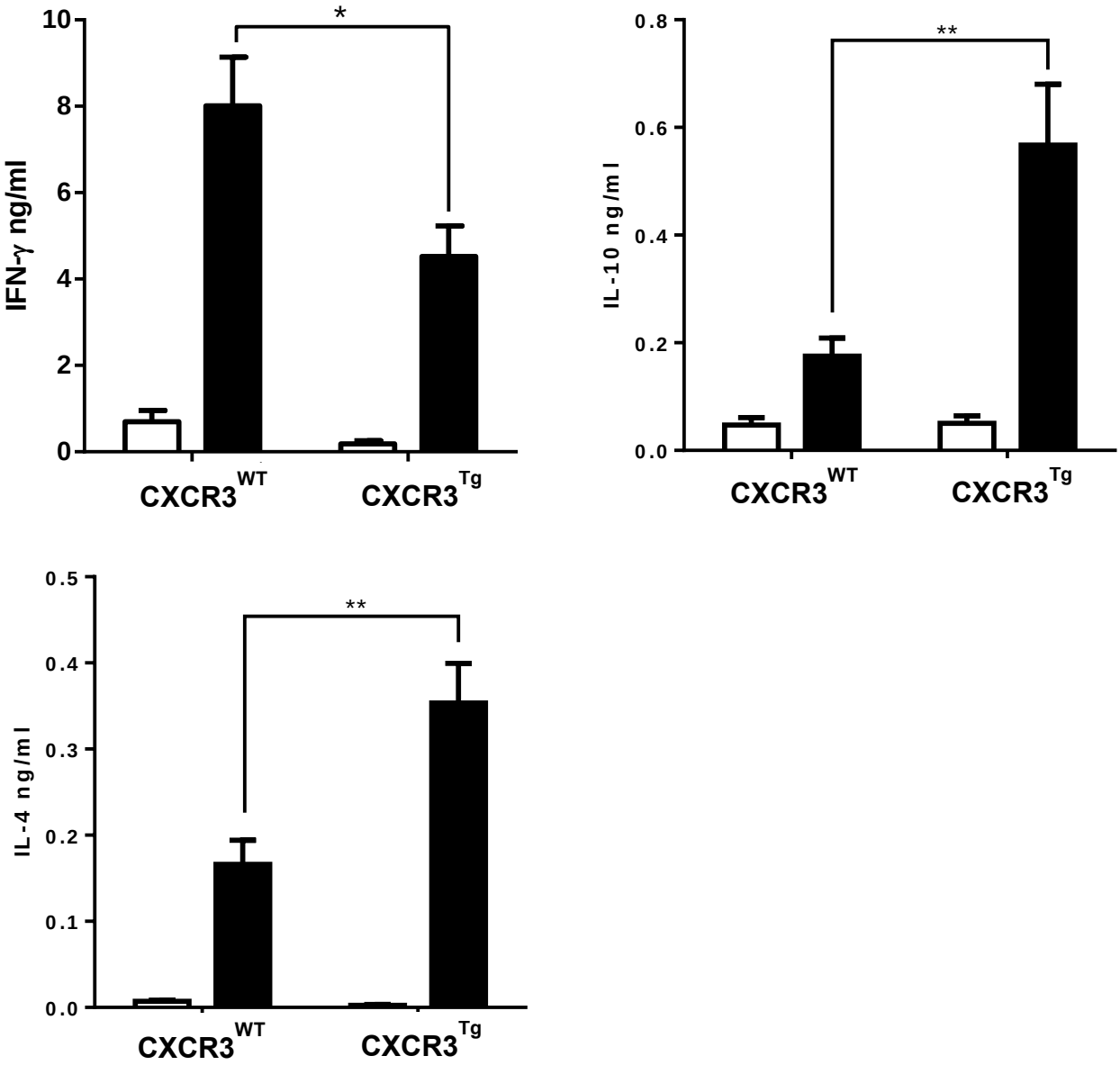

IFN-γ ng/ml
10
8
6
4
2
0
*
CXCR3WT
CXCR3Tg
IL-10 ng/ml
0.8
0.6
0.4
0.2
0.0
**
CXCR3WT
CXCR3Tg
IL-4 ng/ml
0.5
0.4
0.3
0.2
0.1
0.0
**
CXCR3WT
CXCR3Tg

Figure 10

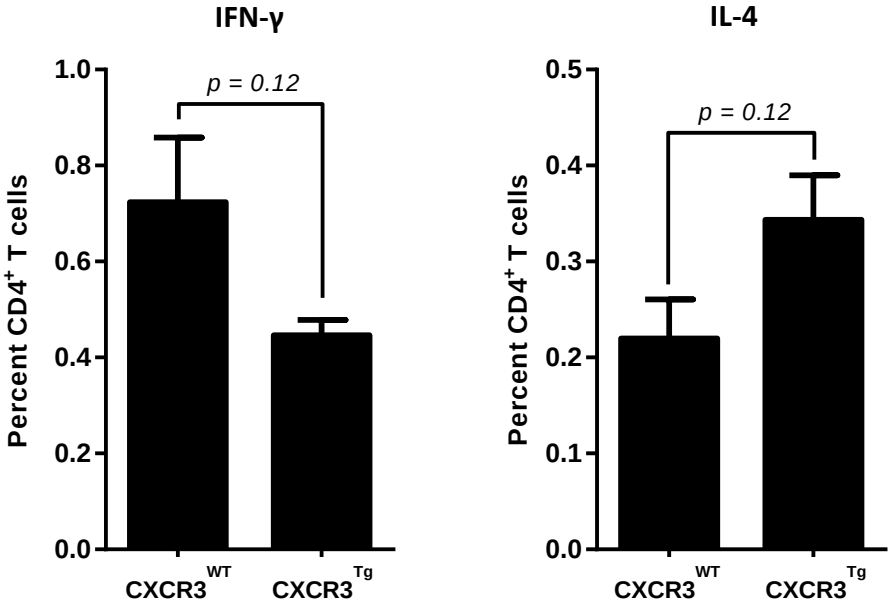


Figure 11

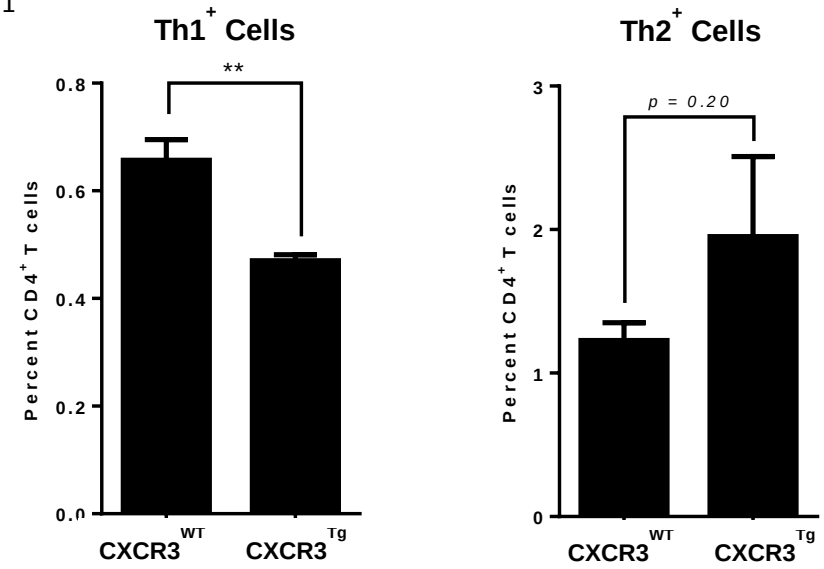


Figure 12

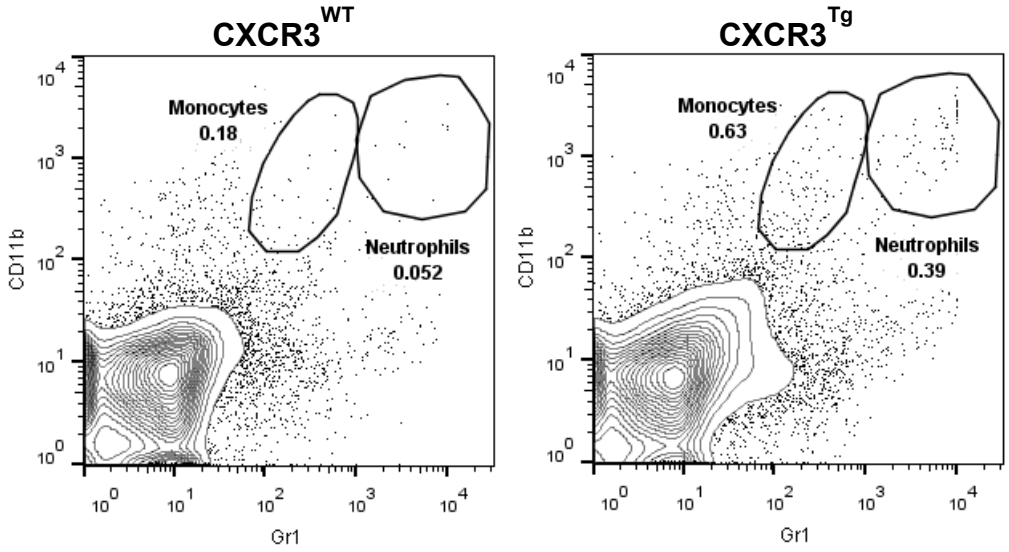


Figure 13

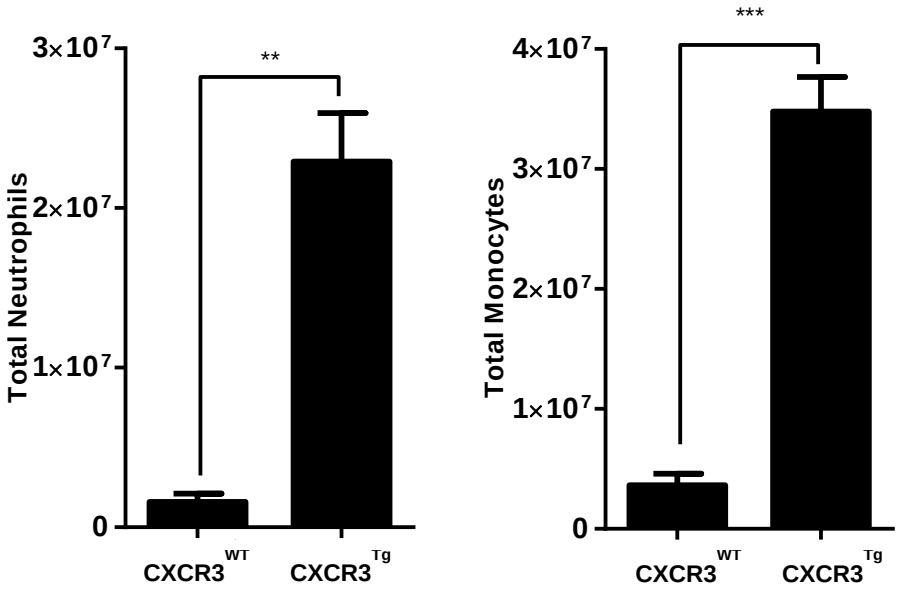


Figure 14

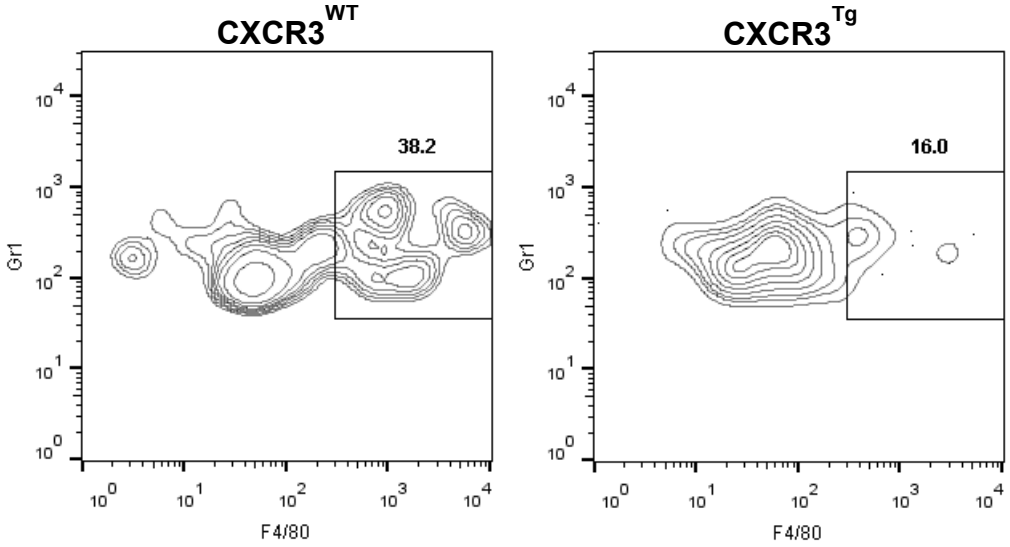
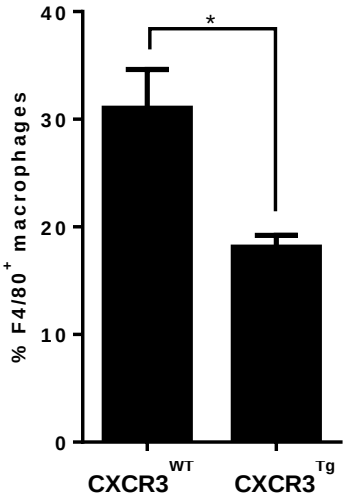


Figure 15



Acknowledgements

This work would not have been possible without the guidance of Dr. Steve Oghumu, Sanjay Varikuti, Dr. Abhay Satoskar, and Dr. Cesar Terrazas. This work was supported by the Ohio State College of Arts and Sciences as well as the Undergraduate Research Office.

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